Morphometric Evaluation of Tumor Matrix Metalloproteinase 9 Predicts Survival after Surgical Resection of Adenocarcinoma of the Lung

Clovis Antônio Pinto,
Paulo Eduardo de Oliveira Carvalho,
Leila Antonângelo, Ana Garippo,
Alecsander Guillauomon Pereira da Silva,
Fernando Soares, Riad Younes, Ricardo Beyruti,
Teresa Takagaki, Paulo Saldiva,
Robin T. Vollmer,2 and Vera Luiza Capelozzi

Department of Pathology [C. A. P., F. S.] and Thoracic Surgery of Hospital do Câncer AC Camargo [R. Y.] and Marília Medical School [P. E. d. O. C.] and Departments of Thoracic Surgery [R. B.], Oncology [T. T.], and Pathology [L. A., A. G., A. G. P. d. S., P. S., V. L. C.], School of Medicine, University of São Paulo, São Paulo, Brazil, and Duke University and VA Medical Centers, Durham, North Carolina 27705 [R. T. V.]

ABSTRACT

Purpose: Recently, several matrix metalloproteinases (MMPs) have shown promise as prognosticators in non-small cell lung cancer. In this study, we sought to validate the importance of MMP-9 and to study the relationships between MMP-9 and several other tumor or stromal markers.

Experimental Design: We examined MMP-9 and several other markers in tumor tissues from 152 patients with surgically excised adenocarcinomas of the lung. Their preoperative clinical stages were T1–4 N0 M0; however, pathological examination of their resected tissues demonstrated that 33 were stage II, and 64 were stage III. We used immunohistochemistry and morphometry to evaluate the amount of tumor staining for MMP-9, and the outcome for our study was survival time until death from recurrent lung cancer.

Results: Multivariate Cox model analysis demonstrated that pathological stage was significantly related to survival time (P < 0.01), but quantitative staining of the tumor for MMP-9 added prognostic information (P < 3.0 × 10−16) and was more strongly prognostic than pathological stage. In the subset of pathological stage I patients, staining for MMP-9 was also significantly associated with survival (P < 1.0 × 10−6), and a cutoff at the median staining of 11.2% for MMP-9 divided them into two groups with distinctive survival times. Those with MMP-9 > 11.2% had a median survival time of just 11 months. Those with MMP-9 < 11.2% had not reached a median survival and had a mean survival time of >62 months.

Conclusions: Tumor staining for MMP-9 in resected adenocarcinoma of the lung is strongly related to survival. Patients with >11.2% staining in their tumors comprise a subset with a high hazard for dying of lung cancer and may be an appropriate target for prospective studies of adjuvant chemotherapy after surgical resection.

INTRODUCTION

A minority of patients with NSCLC1 have tumors sufficiently localized to be considered treatable by surgical resection, and among those whose tumors are successfully resected, approximately 50% survive 5 years (1). Thus, clearly some NSCLCs have developed occult spread beyond the lung even when they appear to have been completely removed. If we could identify those tumors destined to recur, we could treat the patients with adjuvant chemotherapy and perhaps eradicate any residual tumor. Thus there is great interest in ways to identify which tumors are likely to recur and shorten the patient’s life (1, 2), and for the adjuvant treatment to be effective, we must identify these tumors shortly after surgery.

In this regard, many have studied molecular or other markers in the primary tumor as well as the surrounding tissue milieu to discover what might relate to tumor recurrence and shortened survival (1–9). Because degradation of the extracellular matrix has been thought to be important to tumor invasion and metastasis (10, 11), a group of MMPs have been targeted as potentially useful tumor markers (11–27). Among these, MMP-9 has shown promise. MMP-9 is a Mr 92,000 gelatinase that degrades type IV collagen (12), and by IHC, it has been found in 20–65% of NSCLCs (17, 19–25). In some studies, staining for MMP-9 has also been found to be significantly associated with survival (17, 23, 25), but there has been uncertainty about how best to record staining for MMP-9. In fact, there have been nearly as many ways to report IHC for MMP-9 as studies. Some have used binary cutpoints to define a positive tumor. Others have expressed the staining in relation to stromal staining or other proteins, and yet others have expressed it quantitatively. To

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2 To whom requests for reprints should be addressed, at Laboratory Medicine 113, VA Medical Center, 508 Fulton Street, Durham, NC 27705.

3 The abbreviations used are: NSCLC, non-small cell lung cancer; MMP, matrix metalloproteinase; IHC, immunohistochemistry; AgNor, nucleolar organizing regions.
validate the importance of MMP-9 and to explore the quantitative relationship between this factor and outcome as well as the relationship between MMP-9 and other tumor and stromal factors, we studied this marker in 152 cases of localized adenocarcinoma of the lung and herein report our results.

**PATIENTS AND METHODS**

**Patients and Tumor Tissues.** The patients of this study comprised 152 patients with completely resected adenocarcinoma of the lung. All were clinically staged to be T1–4N0M0, and all were considered to have tumors potentially curable by surgical resection. Clinical staging used routine chest X-ray, bronchoscopy, computerized tomography of thorax and upper abdomen, abdominal ultrasound, and bone scan. Mediastinoscopy, computerized tomography of thorax and upper surgical resection. Clinical staging used routine chest X-ray, and all were considered to have tumors potentially curable by carcinoma of the lung. All were clinically staged to be T1 comprised 152 patients with completely resected adenocarcinoma.

**Histological Tumor Variables.** The presence of MMP-9, p53, and Ki-67 was analyzed by immunohistochemical staining using the avidin-biotin immunoperoxidase complex technique, pressure cooking antigen retrieval, biotinylated rabbit antimouse IgG (Dako Corp.; dilution, 1:400), streptavidin combined in vitro with biotinylated horseradish peroxidase (Dako Corp.; dilution, 1:1000), diaminobenzidine tetrahydrochloride, and counterstaining with hematoxylin. The antibodies used were MMP-9 mouse monoclonal clone 56–2A4, which recognizes both latent and active MMP-9 (Biogen; dilution, 1:100), monoclonal mouse antihuman p53 protein (DO7; Dako A/S, Glostrup, Denmark; dilution, 1:40), and Ki-67 antigen (Dako A/S; dilution, 1:1800). Brownish nuclear staining was considered to be evidence of the p53 and Ki-67 antigen expression by cells, whereas membranous and cytoplasmic staining characterized MMP-9 expression. In addition, we quantified the staining as follows. First, at low magnification, we selected the region of highest expression. Then, at ×400, we used an eyepiece systematic point sampling grid with 100 points and 50 lines (Fig. 1) to count the fraction of points overlaying positively stained structures. We averaged this over 10 microscopic fields to obtain a final result as a percentage of staining structures. Examples of tumor staining for p53 and MMP-9 appear in Fig. 1.

In addition, we estimated tumor cell nuclear size as star volume (V* using the stereological method of point-sampled intercepts (29, 30). Briefly, at ×400 magnification, we examined tumor nuclei with an eyepiece sampling grid. For each point in the test system that hit a nuclear profile in focus, the distance from nuclear border to nuclear border was measured through the sampling point in one arbitrary direction. These intercept lengths (L* ) were individually cubed, and an average value over 150 tumor nuclei was multiplied by π/3 to obtain an unbiased volume-weighted estimate of the mean nuclear size as shown below.

\[ V^* = \pi/3 \times L^3 \]

We evaluated AgNOR 100 tumor cells/case using the one-step silver colloid method (31) and a digital image analysis comprising an Axioplan microscope (Zeiss Corp.), a charge-coupled device Sony DXC-101 camera (Trinitron Sony), a digitizer (Oculus TCX; Coreco Inc., St. Laurent, Canada), a Pentium-based computer, and Bioscan-Optimas 5.1 software (Bioscan, Inc., Edmonds, WA). An example of tumor stained for AgNOR also appears in Fig. 1.

**Tissue Environment Variables.** We evaluated microvessel density using the anti-CD34 monoclonal antibody (Novocastra Laboratory, Newcastle, United Kingdom) at a 1:25 dilution and the same immunohistochemical procedure as used for MMP-9, p53, and Ki-67. For each slide, the 10 most vascular areas within the tumor mass were chosen, and at ×200 magnification, we counted the number of CD34-positive staining vascular structures (single cells or cell clusters) by conventional area sampling (29, 30) and then recorded the average counts of the 10 fields. We omitted from the count larger vessels with thick muscular walls or with lumens greater than 50 μm, and we carefully avoided other cells that might stain for CD34 such as transformed lymphocytes.

To evaluate the overall stromal component of tumor, we used point-counting technique and the same eyepiece grid and expressed the result as a ratio of points overlaying stroma to points overlaying tumor cells, averaged over 10 noncoincident microscopic ×250 tumor fields. To evaluate collagen, we used the image analysis system described above, staining by a 0.2% solution of Sirius red (Direct Red 80; C. I. 35780; Aldrich, Milwaukee, WI) in aqueous saturated picric acid and

**Table 1** Patient characteristics

<table>
<thead>
<tr>
<th>Total no. of patients</th>
<th>152</th>
</tr>
</thead>
<tbody>
<tr>
<td>Age (yrs)</td>
<td>61 (22–91)*</td>
</tr>
<tr>
<td>Gender</td>
<td>85 males, 67 females</td>
</tr>
<tr>
<td>Overall stage</td>
<td></td>
</tr>
<tr>
<td>I</td>
<td>55</td>
</tr>
<tr>
<td>II</td>
<td>33</td>
</tr>
<tr>
<td>III</td>
<td>64</td>
</tr>
<tr>
<td>T stage</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>37</td>
</tr>
<tr>
<td>2</td>
<td>68</td>
</tr>
<tr>
<td>3</td>
<td>32</td>
</tr>
<tr>
<td>4</td>
<td>15</td>
</tr>
<tr>
<td>N stage</td>
<td></td>
</tr>
<tr>
<td>0</td>
<td>72</td>
</tr>
<tr>
<td>1</td>
<td>31</td>
</tr>
<tr>
<td>2</td>
<td>41</td>
</tr>
<tr>
<td>3</td>
<td>8</td>
</tr>
<tr>
<td>Subtype of tumor histology</td>
<td></td>
</tr>
<tr>
<td>Acinar</td>
<td>67</td>
</tr>
<tr>
<td>Solid</td>
<td>41</td>
</tr>
<tr>
<td>Bronchioalveolar</td>
<td>44</td>
</tr>
<tr>
<td>Follow-up (months)</td>
<td>16 (6–168)*</td>
</tr>
<tr>
<td>Patients censored for survival analysis at last time of follow-up</td>
<td>34</td>
</tr>
</tbody>
</table>

a Values represent median (range).
polarized light (32–34). We evaluated elastin using the Weigert’s resorcin-fuchsin stain, modified with a previous oxidation (32–34), as well as the same image analysis system. For both collagen and elastin, we analyzed a total of 10 fields/case at ×400 magnification, and we expressed the results as area per unit of stroma; examples of tumors stained for collagen and elastin also appear in Fig. 1.

**Statistical Analysis.** Initial analyses were done using Kaplan-Meier curves, and final multivariate analyses were done using the Cox proportional hazard model (35). In addition, the general linear model was used to test the relationship between one continuous variable and several others, and the residuals were examined to ensure that they were approximately normally distributed. All analyses were done with S-PLUS statistical software.
In this analysis we used a multivariable model. In addition, in significant after allowing for the contribution of the others, and analysis demonstrated that staining for MMP-9 related significantly to several factors having to do with the stage of the tumor, its tumors and in surrounding environment. In addition, we found that the level of staining for MMP-9 related significantly to

...Patients and Methods...

...RESULTS...

Table 2 summarizes the morphometric results found in the tumors and in surrounding environment. In addition, we found that the level of staining for MMP-9 related significantly to several factors having to do with the stage of the tumor, its phenotype, or the tissue environment. General linear model analysis demonstrated that staining for MMP-9 related significantly to overall stage (P = 0.012), to the presence of T1 tumor stage (P = 0.0076), to staining of the tumor for p53 (P < 1 × 10^-7), to nuclear volume (P = 0.0096), and most strongly to vascular density (P < 1 × 10^-8). All these relationships were significant after allowing for the contribution of the others, and for this analysis we used a multivariable model. In addition, in univariate analyses, the level of staining for MMP-9 was related to N stage at a borderline level (P = 0.06). Also, in univariate analysis, MMP-9 was negatively related to the amount of stroma (P = 0.04). In other words, higher levels of tumor staining for MMP-9 were associated with smaller fractions of stroma in the tumor. MMP-9 did not relate to elastin (P > 0.4), collagen (P > 0.5), AgNORs (P > 0.3), or Ki-67 (P > 0.5). Fig. 2 uses four plots to demonstrate the relationships between staining for MMP-9 and T stage (top left), N stage (top right), overall stage (bottom left), as well as stromal vessels (bottom right). The three box plots demonstrate that the relationship between MMP-9 and stage factors was relatively weak. On the other hand, the scatter plot in the bottom right corner shows that there was a strong relationship between staining of the tumor for MMP-9 and stromal microvessel density.

Survival Analysis. Preliminary examination of Kaplan-Meier survival curves (data not shown) demonstrated that in this study, patients with pathological stages II and III had approximately the same hazard for survival with a median survival time equal to 15 months for both groups. Thus, we coded overall pathological stage as a single dummy variable with a value of 0 for stage I and a value of 1 for stages II and III. The results of the Cox model analysis appear in Table 3. Just two variables were significantly associated with survival time: pathological stage and staining of the tumor for MMP-9, which was used as a continuous variable. Once these two variables were accounted for, none of the others related to survival. For example, we found that individual T and N stages were significantly related to survival time in the absence of MMP-9 (P<sub>0.7</sub> ranging from 0.01 to 0.05); however, when MMP-9 was present as a variable, its relationship to survival was much stronger. Whereas the overall likelihood ratio of the Cox model using T and N staging information alone was just 14.8, the likelihood ratio with stage and MMP-9 was 82. After controlling for pathological stage, microvessel density was significantly related to survival (P = 3.8 × 10^-7), but with MMP-9 present as a variable in the Cox model, microvessel density was no longer significant (P > 0.09). The model likelihood ratio with stage and microvessel density as covariates was just 33.5 compared with 82 for stage and MMP-9. We also found that the prognostic information provided by MMP-9 was maximized when this variable was used as a continuous one, that is, one without the usual cutpoint at 20% of the tumor tissue. For example, the Cox model likelihood ratio was just 45 when MMP-9 was used with a cutpoint at 20%, whereas it was 82 when used as a continuous variable. This continuous relationship is also illustrated in Fig. 3. Here, survival time for all those observed to die of recurrent tumor is plotted against MMP-9, and the trend is demonstrated by the loess line smoothing function provided by S-PLUS. The resulting smooth line indicates that there is not a cutpoint in the way survival time depends upon staining for MMP-9.

Finally, we examined the importance of MMP-9 to survival in just the 55 of our patients who had pathological stage I tumors. Once again, MMP-9 was significantly related to survival time by the Cox model (P = 9.8 × 10^-7). In this subset, 50% of the patients had MMP-9 values > 11.2%, and 50% had MMP-9 values ≤ 11.2%. Although there is no biologically distinctive cutpoint in MMP-9, we chose this median value of 11.2% as a practical way to examine the stage I patients’ outcome. We found that it separated these stage I patients into two groups with distinctly different average survival times as illustrated by Kaplan-Meier plots in Fig. 4. The group with ≤11.2% MMP-9 appears as the top curve, and their median survival time was not reached during our follow-up. Their mean survival time, however, was quite long (62.5 months). By contrast, those with >11.2% MMP-9 (bottom curve) had a median survival time of just 11 months after surgery (P < 1 × 10^-6 by log-rank test).

DISCUSSION

Clearly, the likely reason that surgical excision fails to cure some patients with localized NSCLC is because of occult metastases that have not yet been detected by either routine imaging or routine pathological analysis. The question of interest is whether additional, more technological information gathered from either the tumor tissue or its milieu can help us identify tumors likely to have occult metastases. The process of cancer cell invasion and metastasis undoubtedly comprises a series of complex, sequential steps, but among these the proteolysis of basement membranes and other extracellular matrix by either tumor cells or endothelial cells is thought to be important.

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**Table 2** Summary of morphometric results* |

<table>
<thead>
<tr>
<th>Tumor variables</th>
<th>Mean</th>
<th>Range</th>
</tr>
</thead>
<tbody>
<tr>
<td>MMP-9 (% of points)</td>
<td>11.4</td>
<td>0.2–29.2</td>
</tr>
<tr>
<td>p53 (% of points)</td>
<td>14.6</td>
<td>0.0–67.0</td>
</tr>
<tr>
<td>Ki-67 (% of points)</td>
<td>4.0</td>
<td>0.0–18.2</td>
</tr>
<tr>
<td>Nuclear volume (μm²)</td>
<td>12.1</td>
<td>0.3–45</td>
</tr>
<tr>
<td>AgNOR (μm²)</td>
<td>9.0</td>
<td>3.2–19.6</td>
</tr>
</tbody>
</table>

*The units of “% of points” indicate the number of points overlying the phenomena of interest divided by the total number of points overlying tumor. In morphometry, this is called a point fraction and is often symbolized as Pp. Pp has been shown to approximate the volume fraction or Vv (29). The units for elastin and collagen are log(area) with the area determined by optical density (see “Patients and Methods”).

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because this breakdown facilitates the migration of tumor cells and penetration of tumor by blood vessels (10, 11). The MMPs belong to a class of transmembrane or secreted endopeptidases that degrade components of the extracellular matrix, and recent studies have shown that several MMPs are increased in the early stages of tumor progression. In a variety of tumors, increased expression of MMPs as detected by IHC has been associated with unfavorable outcomes (36–38). Among these, MMP-2 and MMP-9 have been especially noted. Thus, for all these reasons, we should not be surprised to learn that IHC staining for MMPs provides important prognostic information about NSCLC, and our results now confirm the prognostic importance of MMP-9 in adenocarcinomas of the lung. Whereas only three prior studies were able to show a significant relationship between IHC staining for MMP-9 in the tumor and survival (17, 23, 25), our results suggest that staining for MMP-9, used as a continuous variable, provides more prognostic information than does routine pathological stage. Furthermore, the prognostic importance of MMP-9 persisted in the subset of patients with pathological stage I. Although we were unable to identify a binary cutpoint in MMP-9, a natural dividing point these stage I tumors was the median of 11.2% of cells staining for MMP-9, and this point provided a practical way to separate them into two groups: patients with an expected short survival versus patients with an expected longer survival. Thus, IHC staining of the primary tumor for MMP-9 offers us the potential to guide the use of adjuvant chemotherapy in patients likely to fail after surgical excision of NSCLC. To finalize this conclusion will require greater study in a randomized and prospective trial, and we also believe it important to validate our quantitative assessment of MMP-9 as well as to extend it to other histological types of NSCLC by studying MMP-9 in additional patients.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Coefficient</th>
<th>SE</th>
<th>P</th>
</tr>
</thead>
<tbody>
<tr>
<td>Stage &gt; 2</td>
<td>0.543</td>
<td>0.207</td>
<td>0.0087</td>
</tr>
<tr>
<td>MMP-9</td>
<td>0.130</td>
<td>0.0157</td>
<td>2.2 × 10⁻¹⁶</td>
</tr>
</tbody>
</table>

In this analysis, MMP-9 was used as a continuous variable with units of percent. SE stands for the SE of the coefficient.

Fig. 2 Four plots demonstrate the relationships between staining for MMP-9 and T stage (top left), N stage (top right), overall stage (bottom left), as well as stromal vessels (bottom right). In the box plots, the solid bar represents the values of MMP-9 between the 25th and 75th percentiles; the white bar shows the median value; and the top and bottom brackets show the extreme values.

Fig. 3 Plot of natural logarithm of survival time for all those observed to die of recurrent tumor on vertical axis versus percentage of tumor cells staining for MMP-9 on the horizontal axis. The trend in the points is given by the line, which comes from the loess line smoothing function of S-PLUS.
amount of stroma, but once again MMP-9 provided more prognostic information. Most interesting was the strong, quantitative relationship we found between MMP-9 and microvessel density. MMP-9 was positively associated with vessel density, even after controlling for other, related variables. Although microvessel density has been repeatedly found to be a strong prognostic variable in resectable NSCLC (39–44), we found that its relationship with survival was not as strong as that for MMP-9. Using microvessel density as a covariate with stage in the Cox model produced an overall likelihood ratio of 33.5, whereas using MMP-9 and stage resulted in a likelihood ratio of 82. Because the likelihood ratio was greater when using MMP-9, we conclude that MMP-9 provided more prognostic information in our patients than did microvessel density.

A variety of associations between MMPs and angiogenesis have been described recently (45). Not only have MMPs been thought to break down connective tissue and thus allow vascular migration, but they have also been thought to regulate endothelial cell attachment and proliferation (45). Hiraoka et al. (46) also showed that MMPs in endothelial cells regulate neovascularization. In ovarian tumors, Gazzetti et al. (47) found that staining of tumor cells for vascular endothelial growth factor correlated to staining for MMP-2. In addition, Bergers et al. (48) found that expression of both MMP-2 and MMP-9 was increased in pancreatic islets that had become angiogenic and that MMP-9 could make normal islets angiogenic as well as release vascular endothelial growth factor. Now, we have demonstrated that in pulmonary adenocarcinoma there is a strong correlation between microvessel density and tumor level of MMP-9, and to the best of our knowledge, this is the first report of this association in NSCLC. The strong association between tumor staining for MMP-9 and angiogenesis suggests that tumors manufacturing and secreting higher levels of MMP-9 facilitate growth and penetration of capillaries into the tumor tissue milieu. Thus, increased tumor expression of MMP-9 may be more of a primary event, and increased angiogenesis may be more of a secondary event. Regardless of the mechanism, staining of the primary tumor for MMP-9 provides important prognostic information in adenocarcinomas of the lung after surgical resection.

Fig. 4 Kaplan-Meier plots of survival probability versus follow-up time in months in those patients with pathological stage I disease. The group with ≤11.2% MMP-9 appears as the top curve, and the group with >11.2% MMP-9 appears as the bottom curve.

REFERENCES


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